Defensin

Week 16

Summarized below are the experiments conducted this week in chronological order. Click on the experiment name to view it. To go back to this summary, click **Summary** in the footer.

Summary

1 IPTG induction of pSB1C3-T7-Def plasmid containing BL21(DE3) cells with protein extraction and SDS-PAGE analysis

Responsible

Oscar He and Aman Mebrahtu

Protocols used

Protein Expression
Sonication
SDS-PAGE

Modifications and comments to protocols

	Def	no IPTG
Protein expression (samples):	Def	0.5 mM IPTG
	Def	1.0 mM IPTG

SDS-PAGE gel: Mini-PROTEAN®TGX™ precast gel

Experimental Set Up

Table 1: Volumes of each liquid culture. From some, 1 ml was taken for optical density measurement at 660 nm.

Sample	Volume [ml]
Def - no IPTG	20
Def - 0.5 mM IPTG	20
Def - 1.0 mM IPTG	20

Results and Conclusions

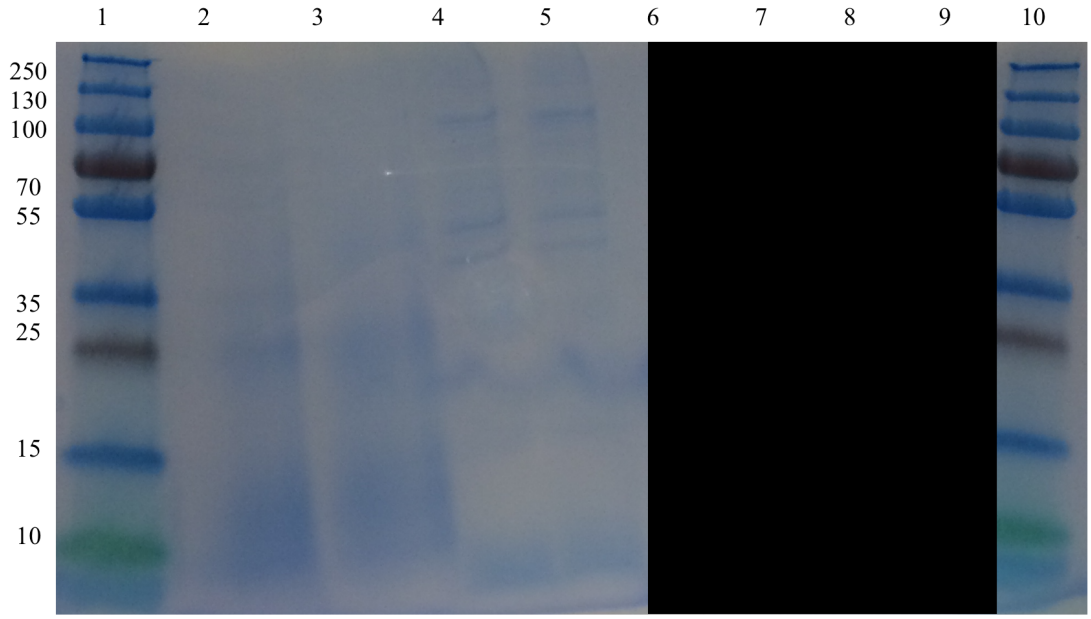

Figure 1: Result of the SDS-PAGE run at 200 V, 3.00 A. (1) Protein ladder (2) Def1 1.0 mM supernatant (3) Def2 1.0 mM supernatant (4) Def1 1.0 mM pellet (5) Def2 1.0 mM pellet.

No bands visible at the expected size of 10.7 kDa.

Discussion and Troubleshooting

The absence of visible bands corresponding to defensin could be due to a too low concentration of the protein in the cell lysate. However, there could be another reason. Besides bands corresponding to the protein of interest, there is also expected to be a substantial amount of other bands corresponding to the proteins normally present in the bacterial cells. The absence of most of these proteins and defensin indicates that the sonication might have been to harsh and degraded most of the proteins.

2 Addition of BamHI restriction site downstream to the defensin sequence and amplification of the whole plasmid, using overhang PCR for further use

Responsible

Oscar He

Protocols used

PCR Amplification (Phusion)

Modifications and comments to protocols

PCR Amplification (Phusion) primers:	Def_R (reverse) Suffix_F (forward)
PCR Amplification (Phusion) buffer:	GC Buffer
PCR Amplification (Phusion) duplicates:	5

Experimental Set Up

Table 2: Volumes of components in PCR mix (with DMSO).

Component	Volume μl]
10 μM Suffix_F	1
10 μM Def_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
DMSO	0.6
ddH_2O	10.2

Table 3: Thermocycling conditions, with two different annealing temperatures.

Step	Temperature [°C]	Time [sec]
Initial Denaturation	98	30
35 x	98	10
	65	30
	72	60
Final Extension	72	600
Hold	4	N/A

Results and Conclusions

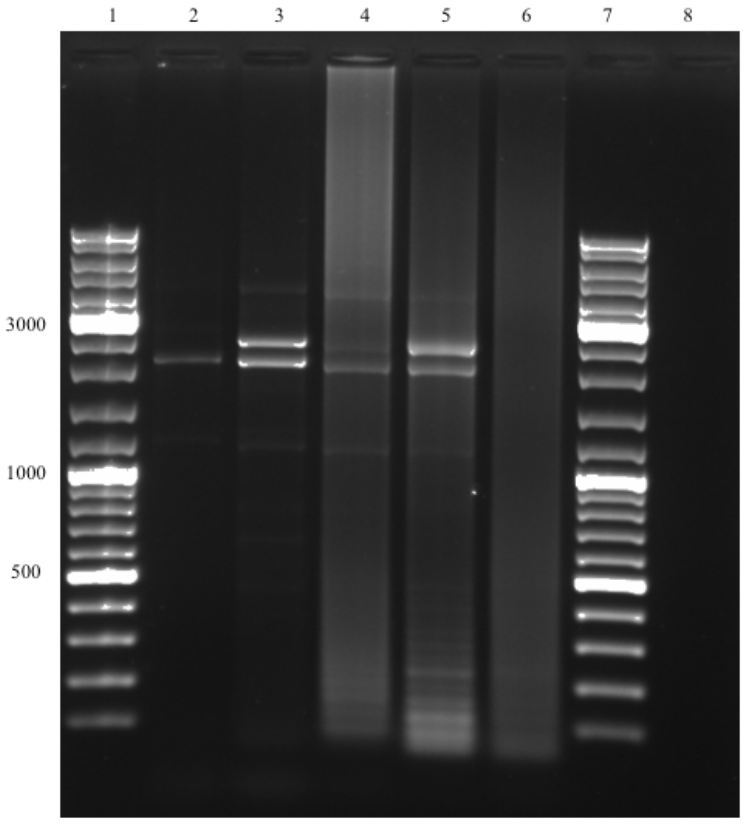

Figure 2: Result of the gel electrophoresis of overhang PCR product, with 1 % agarose at 110 V. (1) DNA Ladder (2) Def (3) Def (4) Def (5) Def (6) Negative control (7) DNA Ladder.

Discussion and Troubleshooting

Since only one of the sample was successful, the samples were not pooled for further use. This was unexpected, since the same PCR program as in the second attempt was used. The decision was made to only continue with the successful sample from the second attempt.

3 Extraction of pSB1C3-T7-Def plasmid from Top10 cells

Responsible

Oscar He

Protocols used

Plasmid Extraction

Modifications and comments to protocols

Plasmid Extraction:	LyseBlue reagent added to PI Buffer
Plasmid Extraction - Step 1:	centrifugation at 13,000 rpm ($\sim$ 17,000 x g)
Plasmid Extraction - Step 6:	repeated 2 times
Plasmid Extraction - Step 7:	not performed
Plasmid Extraction - Step 10:	DNA elution using EB Buffer

Sample Calculation

The final concentration of plasmid sample in PCR mix

$$Final\ concentration = \frac{Initial\ concentration}{Dilution\ factor} \tag{1}$$

Results and Conclusions

Table 4: Concentration of extracted plasmid sample measured using NanoDrop at 280 nm.

Sample	Concentration [ng/μl]
Def Top10 1.1	29.3
Def Top10 1.2	39.8
Def Top10 2.1	30.4
Def Top10 2.2	29.9

Discussion and Troubleshooting

No PCR analysis was performed due to time limits.

4 Digestion of PCR (Phusion) product from the second attempt and linker-tag sample with PCR purification.

Responsible

Oscar He

Protocols used

3A Assembly
PCR Purification

Modifications and comments to protocols

3A Assembly: total volume 20 μl (digestion mix)
3A Assembly: volume BamHI enzyme 2.0 2.0 μl

Experimental Setup

Table 5: Concentration of the pSB1C3-T7-Nuc-BamHI linear plasmid PCR (Phusion) product and linker tag sample used for digestion.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc-BamHI	425.5
Linker-tags (10 μM)	351.6

Table 6: Volumes of components in the digestion mix.

Component volumes	pSB1C3-T7-Nuc-BamHI [μl]	Linker-tags [μl]
DNA sample	2.5	2.84
10x SuRE/Cut Buffer H	2.0	2.0
PstI	1.0	1.0
BamHI	2.0	2.0
ddH_2O	12.5	12.16

Table 7: Volume taken for PCR purification.

Sample	Volume [μl]
pSB1C3-T7-Nuc-BamHI	20 (all)
Linker-tags	20 (all)

Sample Calculation

The volume of DNA sample to add to the digestion mix

$$Volume\ of\ DNA\ sample = \frac{Final\ amount\ of\ DNA}{Concentration\ of\ DNA\ in\ sample} \quad (2)$$

Results and Conclusions

Table 8: The concentration of the sample after PCR Purification, measured with NanoDrop at 280 nm.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc-BamHI	8.5
Linker-tags	38.5

Discussion and Troubleshooting

It is not possible to obtain any useful results from a gel electrophoresis analysis, hence it is not performed. The choice of Buffer SuRE/Cut Buffer was according to the double digest table on Roche website. The activity of BamHI is less than 50 % in SuRE/Cut Buffer H, so the volume for BamHI restriction enzyme was doubled.

5 3A assembly (ligation) of pSB1C3-T7-Def-BamHI-Tags with PCR analysis

Responsible

Oscar He

Protocols used

3A Assembly
PCR Amplification (Taq)

Modifications and comments to protocols

3A Assembly:	Amount of vector per reaction	50.0 ng
	Ratio vector:insert	1:10
	Incubation	over night at 4 °C
PCR Amplification (Taq) primers:		VF2_R (reverse)
		VF2_F (forward)

Experimental Set Up

Table 9: Concentration and size of the parts used for the ligation in the 3A assembly.

Assemble part	Concentration [ng/μl]	Size [bp]
pSB1C3-T7-Def-BamHI	8.5	2273
Linker-tags	38.5	84

Table 10: Volumes of each component used in the ligation mix.

Component	Volume [μl]
pSB1C3-T7-Nuc-BamHI	5.88
Linker-tags	0.46
T4 DNA Ligase	1
10x T4 DNA Ligase Buffer	2
ddH_2O	10.64

Sample Calculation

Moles of vector and mass of insert per reaction were calculated with NEBioCalculator^{TM}.

Volume of pSB1C3, T7 and defensin

$$Volume\ of\ vector = \frac{Amount\ of\ vector\ per\ reaction}{Concentration\ of\ vector\ sample} \tag{3}$$

$$Volume\ of\ insert = Ratio\ \cdot\ \frac{Amount\ of\ vector\ per\ reaction}{Concentration\ of\ insert\ sample} \tag{4}$$

Results and Conclusions

No analysis was performed on the ligation product.

Discussion and Troubleshooting

The ligation was assumed to be successful since the subsequent transformation turned out to be successful.

6 Transformation of pSB1C3-T7-Def-BamHI-Tags into Top10 and BL21(DE3) cells and inoculation in liquid media

Responsible

Aman Mebrahtu

Protocols used

Transformation
Colony picking

Modifications and comments to protocols

Transformation recovery time: 2 h

Results and Conclusions

Transformation successful. Colonies for both Top10 and BL21(DE3). Only one colony of transformed BL21(DE3).

7 IPTG induction of pSB1C3-T7-Def-BamHI-Tags transformed BL21(DE3) cells with protein extraction, IMAC purification, and SDS-PAGE analysis

Responsible

Aman Mebrahtu, Oscar Frisell and Ellinor Lindholm

Protocols used

Protein expression
Sonication
SDS-PAGE
IMAC Purification

Modifications and comments to protocols

	Def-Tags	no IPTG
Protein expression (samples):	Def-Tags	0.5 mM IPTG
	Def-Tags	1.0 mM IPTG

SDS-PAGE gel: Mini-PROTEAN®TGX™ precast gel

Experimental Set Up

For the IMAC purification, the purified Def-Tags samples were prepared by resuspending the pellet in 4 ml Binding buffer (Buffer A) while keeping the supernatant kept in the lysis buffer. This resulted in, when purifying using IMAC, that the chelating agent was eluted as the protein was added (since the lysis buffer is not compatible with the IMAC column). The non-packed PD-10 IMAC column was used to change the buffer by adding the eluate again, washing it, and eluting it three times, 1.5 ml each in Eppendorf tubes, using the binding buffer instead. NanoDrop was used to determine if the protein was present in the new buffer or not.

Table 11: Concentration of Def-Tags protein after buffer exchange.

Sample	Concentration [mg/ml]
1	0.35
2	0.73
3	1.82

The concentrations can be seen in Table 11. For the pellet samples, no protein was present when measuring absorbance using NanoDrop. The supernatant samples were then all added to the same, prepared IMAC column for purification. However, this time the column was again affected by the buffer and some of the chelating agent was eluted. All the eluates before adding elution buffer (Buffer C) and after adding washing buffer (after addition of protein) were kept to make sure the protein was not discarded.

Results and Conclusions

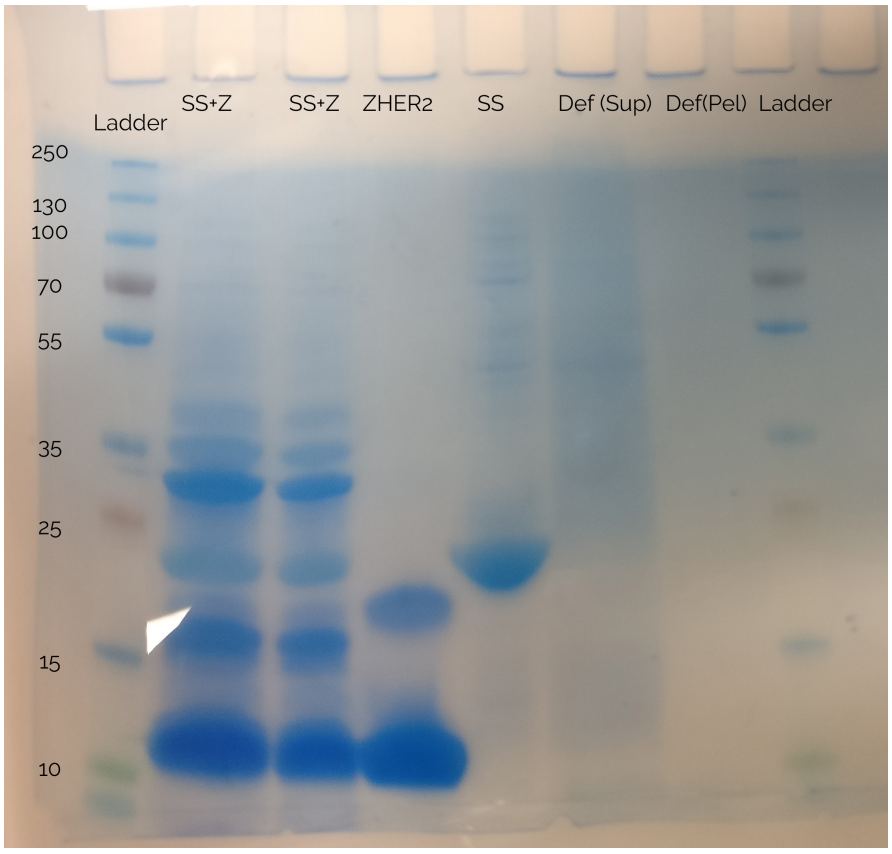

Figure 3: SDS-PAGE results from the supernatant and the pellet of the cell lysate.

The SDS-PAGE did not show any bands at all for the pellet resuspension sample and only very faint bands for the supernatant sample. Probably, the concentrations were too low.

Table 12: Concentration of Def-Tags protein after IMAC. The 'Def-elute before wash' sample refers to the elution samples after adding the protein. The 'Def-wash' refer to after adding the protein and washing the column with Binding buffer (Buffer A) to remove non-specific proteins. Lastly, the 'Def-elute' samples refer to the eluted samples using Elution buffer (Buffer C), where the protein is expected to be present. Elution buffer is then used as a blank when measuring the concentration of the elute samples, and Binding buffer is used as a blank when mesuring before wash and after wash.

Sample	Concentration [mg/ml]
Def-elute before wash	1.20
Def-wash 1	9.46
Def-wash 2	7.22
Def-wash 3	0.05
Def-wash 4	-0.00
Def-wash 5	0.05
Def-wash 6	0.15
Def-elute 1	-0.95
Def-elute 2	-0.47
Def-elute 3	-0.02
Def-elute 4	-0.01
Def-elute 5	-0.02
Def-elute 6	0.00

Discussion and Troubleshooting

When sonication is used as a cell lysis method, one often adds lysis buffer to increase the efficiency of cell lysis. However, the lysis buffer is not compatible with the IMAC-column being sensitive to very low or high pH-values. Because the buffer was not changed before adding it to the column, the IMAC column was damaged. However, the protein was still captured and could be used for a second IMAC purification after the buffer exchange. The reason for damaging the column even this time could be because some of the lysis buffer was left in the protein samples. As the protein concentration was measured as zero for the pellet samples, it was concluded that the protein was not associated with the pellet or membrane but soluble which was expected.
For the purification of Def-Tags using the new buffer, the concentrations can be seen in Table 12. To start with, the concentrations of the wash samples are not to trust as they include parts of the eluted resin which results in false concentration values. For that reason, none of the wash samples will be used further. For the 'Def-elute before wash' sample, the concentration might be true as the protein might not have bound to the resin and thus has eluted immediately. This samples is a candidate to be used for the conjugation. For the elution samples (where the protein of interest should be in theory), the concentrations are obviously not of any use and the negative concentrations can be a consequence of at this point having a mixture of buffers in the eluate making the blank incorrect when measuring the concentration. Especially as the concentration increases, which can be a sign of protein being present, but measured in a buffer C background (although the first elutions will be in the previous buffer). It can be worth measuring the absorbance using buffer A as a blank.

8 Kirby-Bauer test on expressed defensin (pSB1C3-T7-Def)

Responsible

Reskandi Rudjito

Protocols used

Kirby-Bauer test

Modifications and comments to protocols

Protein/antibiotic samples:	Kanamycin	35 mg/ml (positive control)
	dH_2O	(negative control)
	Def SN	0.5 mM IPTG
	Def SN	1.0 mM IPTG
	Def P	0.5 mM ITPG
	Def P	1.0 mM ITPG
Incubation time		16 h
Volume of TOB1 cells on McConkey plates		200 μl

Experimental Set Up

Two plates were prepared with Def supernatant (SN) and Def pellet (P) respectively. Kanamycin was used as a positive control and dH_2O as a negative control on both plates. Whatman papers were impregnated with 200 μl of sample, positive control, and negative control.

Results and Conclusions

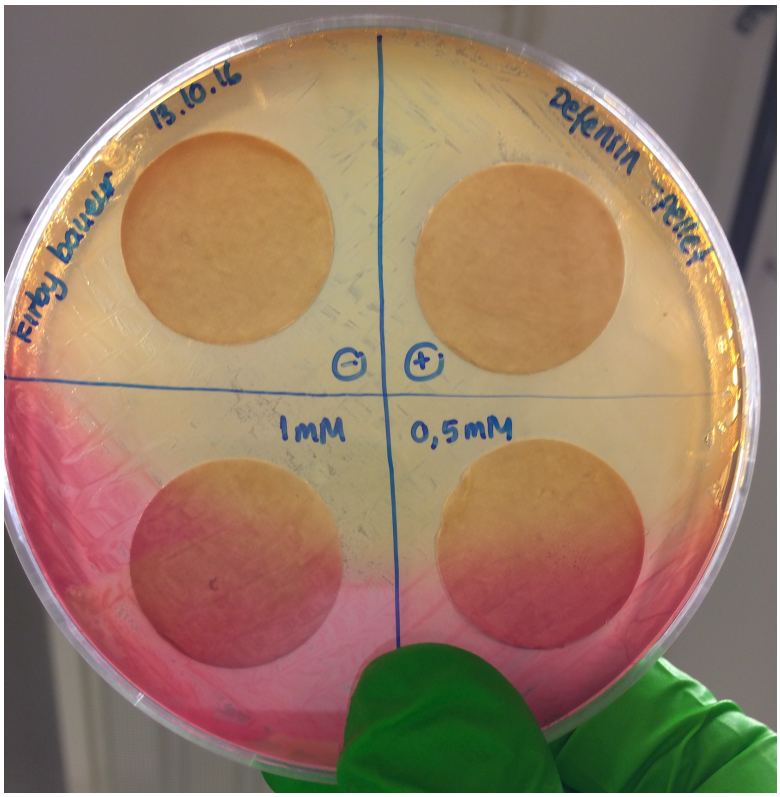

Figure 4: Result of the incubation of TOB1 cells on MacConkey agar with impregnated Whatman paper discs. Pellet sample.

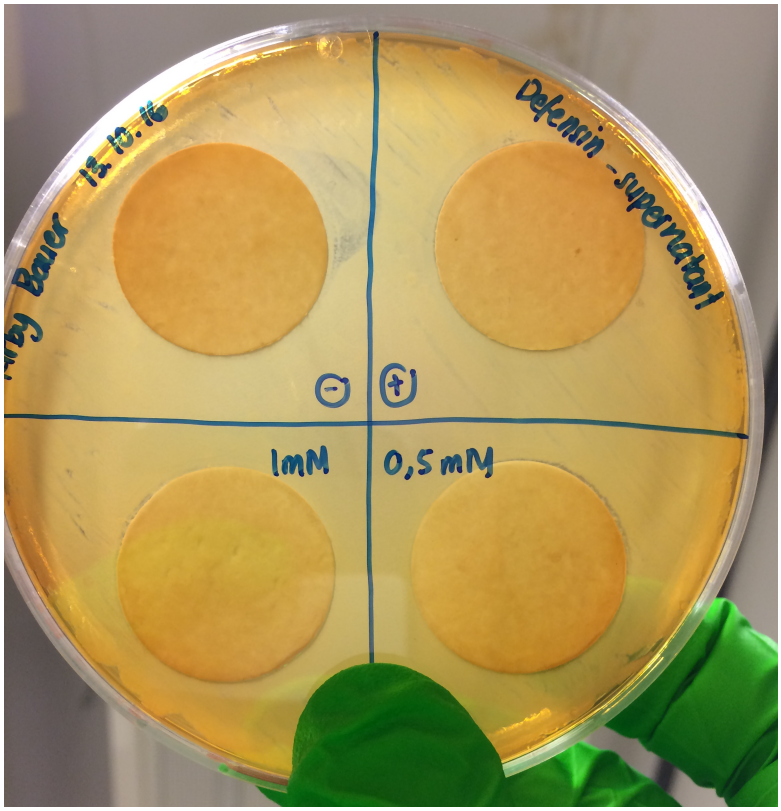

Figure 5: Result of the incubation of TOB1 cells on MacConkey agar with impregnated Whatman paper discs. Supernatant sample.

Discussion and Troubleshooting

No impeded growth of cells could be seen in the Kirby-Bauer tests neither the supernatant samples nor the pellet samples. Most likely, the concentration of defensin in the samples used were too low to show the desired activity on the test. The same samples were used for the biofilm assay, which gave more precise and reliable data regarding the activity of defensin.

9 Biofilm assay on pSCB1C3-T7-Def

Responsible

Shuangjia Xue, Jenny Waspe, and Aliki Mitropoulou

Protocols used

Biofilm Assay

Experimental Set Up

To demonstrate biofilm dispersal by defensin a crystal violet assays is used, one with *P. aeruginosa* as the biofilm-producer and the other with *S. aureus*. For the experiment, a combination of pellet resuspension and supernatant with the ratio 1:1 was used, e.g. for a 5 μl treatment, 2.5 μl of each is used. The biofilm was treated with 5 μl, 20 μl or 50 μl of defensin. Only one volume of negative control is used. Differences between the negative control and Def is evaluated using the students t-test. A p-value ¡0.05 is considered statistically significant. The samples are labelled "Def" and the negative control is labelled "Neg".

A crystal assay was performed to evaluate the inhibition efficiency of defensin on biofilm growth by *P. aeruginosa* and *S. aureus*, respectively. Induced BL21(DE3) cell lysate samples (both debris pellet resuspension and supernatant) are used for the experiment.

S. aureus is cultured in BHI media and *P. aeruginosa* PAO in LB media. 200 μl of the liquid culture is added into each well and treated with defensin of three different volumes; 5, 20 or 50 μl. The biofilm is allowed to develop for 27 hours with subsequent staining. OD is then measured at 595 nm. Cell lysates of BL21(DE3) cells are used as negative control. The samples from supernatants and pellet are labelled "Def S" and "Def P", respectively.

Results and Conclusions

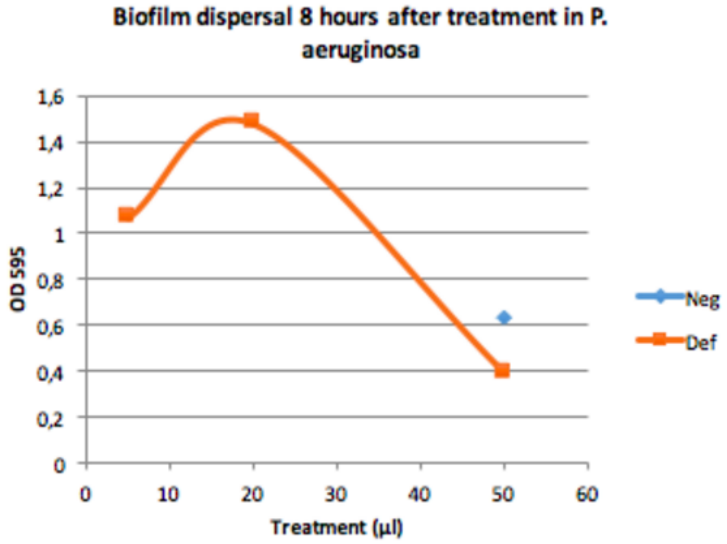

Figure 6: OD measured for different treatments of Def using *P. aeruginosa* as biofilm-producer.

The graph illustrates how defensin (in a combination of pellet and supernatant) dispersed biofilm, depending on the amount of treatment. There is a significant difference between the negative control and each of the treatment volumes. However, the negative control also seems to disperse biofilm. This may be due to contamination or due to the large amount of treatment ($50\,\mu$l), and thus the results may not be reliable.

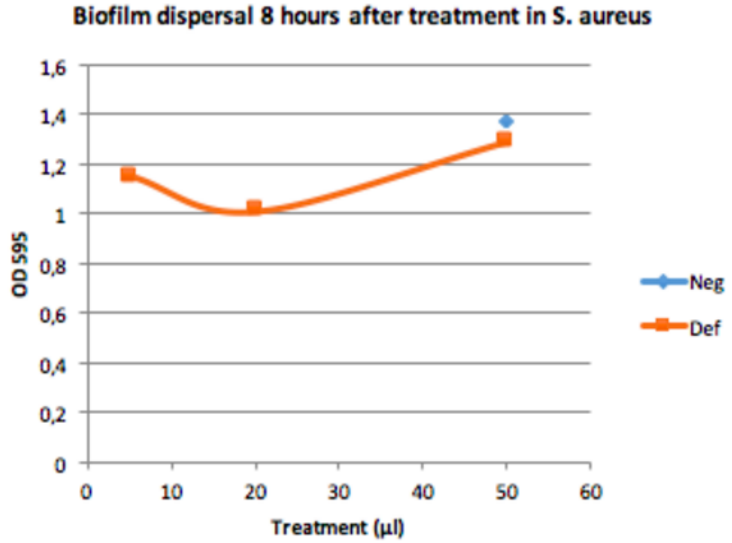

Figure 7: OD measured for different treatments of Def using *S. aureus* as biofilm-producer.

The graph illustrates how defensin (combination of pellet and supernatant) dispersed biofilm by *S. aureus*. There is a significant difference between negative control and 5 μl and 20 μl of treatment, respectively.

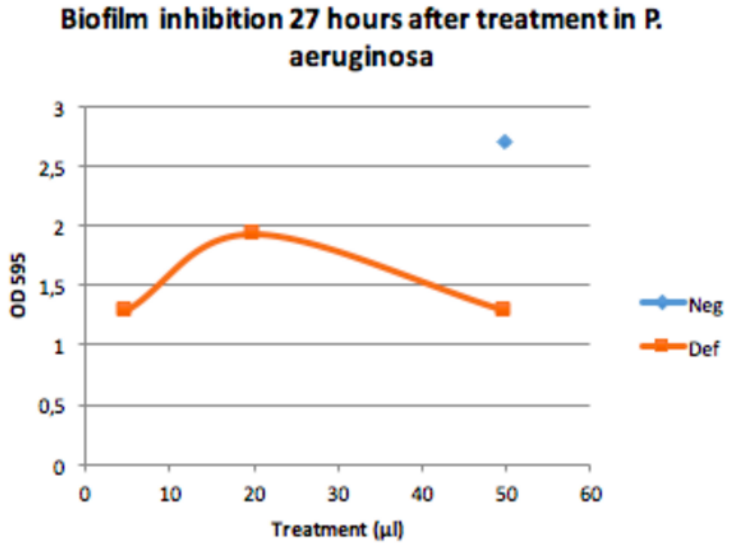

Figure 8: OD measured for different treatments of Def using *P. aeruginosa* as biofilm-producer.

The graph shows how defensin (combination of pellet and supernatant) inhibited biofilm formation in P. aeruginosa. There is a significant difference between the negative control and 5 μl and 50 μl of treatment with defensin, respectively.

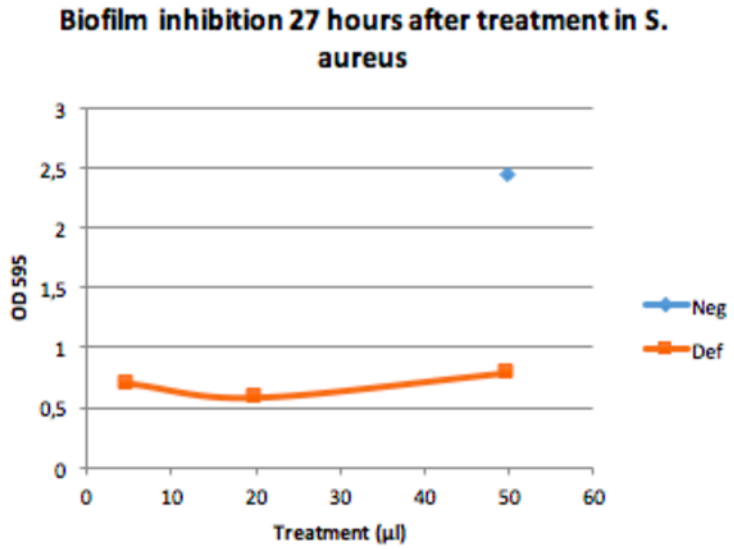

Figure 9: OD measured for different treatments of Def using *S. aureus* as biofilm-producer.

The graph shows how defensin inhibited biofilm-formation in *S. aureus*. There was a significant difference between the negative control and each of the three volumes of treatment with defensin, thus proving defensin as a potential combat protein for the inhibition of biofilm formation in *S. aureus*.

10 Spider silk protein conjugation of His_6-tag and cognition motif (LPETGG) fused defensin using Sortase A with SDS-PAGE analysis

Responsible

Oscar He

Protocols used

Sortase A Conjugation
SDS-PAGE

Modifications and comments to protocols

Sortase A Conjugation total volume:	400 μl
SDS-PAGE gel:	Mini-PROTEAN®TGX™ precast gel

Experimental Set Up

Table 13: Volumes of components used in the conjugation reaction mix.

Component	Volume [μl]	Size [kDa]
Spider silk	231	23
Defensin	107	10.7
10x Sortase A Binding Buffer	40	-
5 μM Sortase A	2	-
dH_2O	20	-

There was not enough spider silk, so the volume of 231 was not reached.

Sample Calculation

Amount of defensin = 107 μl $\Rightarrow$
$(c_2 * V_2)/(c_1)=(n_2)/(c_1)=V_1$=120nmol/(1.2[mg/ml]/10.7[kDa]) = 107 μl.
Amount of Sortase A Binding buffer= 55μl $\Rightarrow$
1x in final volume, 10x in stock,
(1x/10x)*Total volume= 0.1*400= 40μl
Amount of Sortase A = 2.75 μl $\Rightarrow$
V_1=(5μM*400μl)/1mM=2.75μl
Amount of Spider silk with N-terminal= 231μl $\Rightarrow$
M_w=22.997kDa
Concentration = 3 mg/ml= 130 nmol/ml
Volume = (30nmol)/ (139nmol/ml) = 231 μl

Results and Conclusions

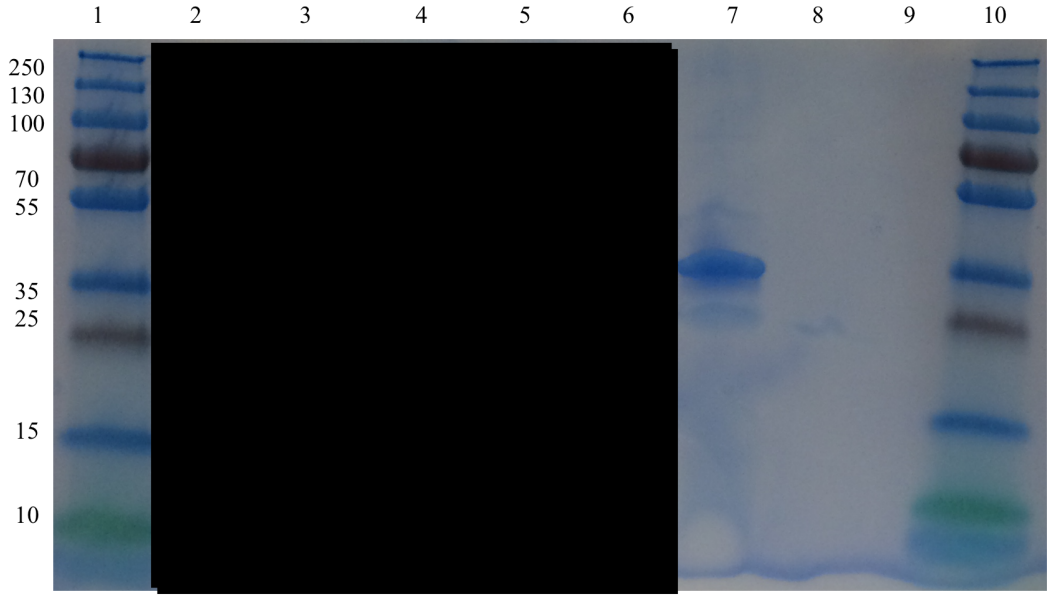

Figure 10: Result of the SDS-PAGE run at 200 V, 3.00 A. (1) Protein ladder (7) Spider silk with Def (8) - (9) Spider silk reference (10) Protein ladder.

A clear band at the expected size of approximately 33.7 kDa, corresponding to defensin-fused spider silk protein, can be seen in well 7. A faint band just below that band, at an approximate size of 23-24 kDa is visible and probably corresponds to spider silk protein not fused with defensin. The non-fused spider silk reference in well 9 is not visible, which is most likely due to the very small amount added as the total amount was limited.

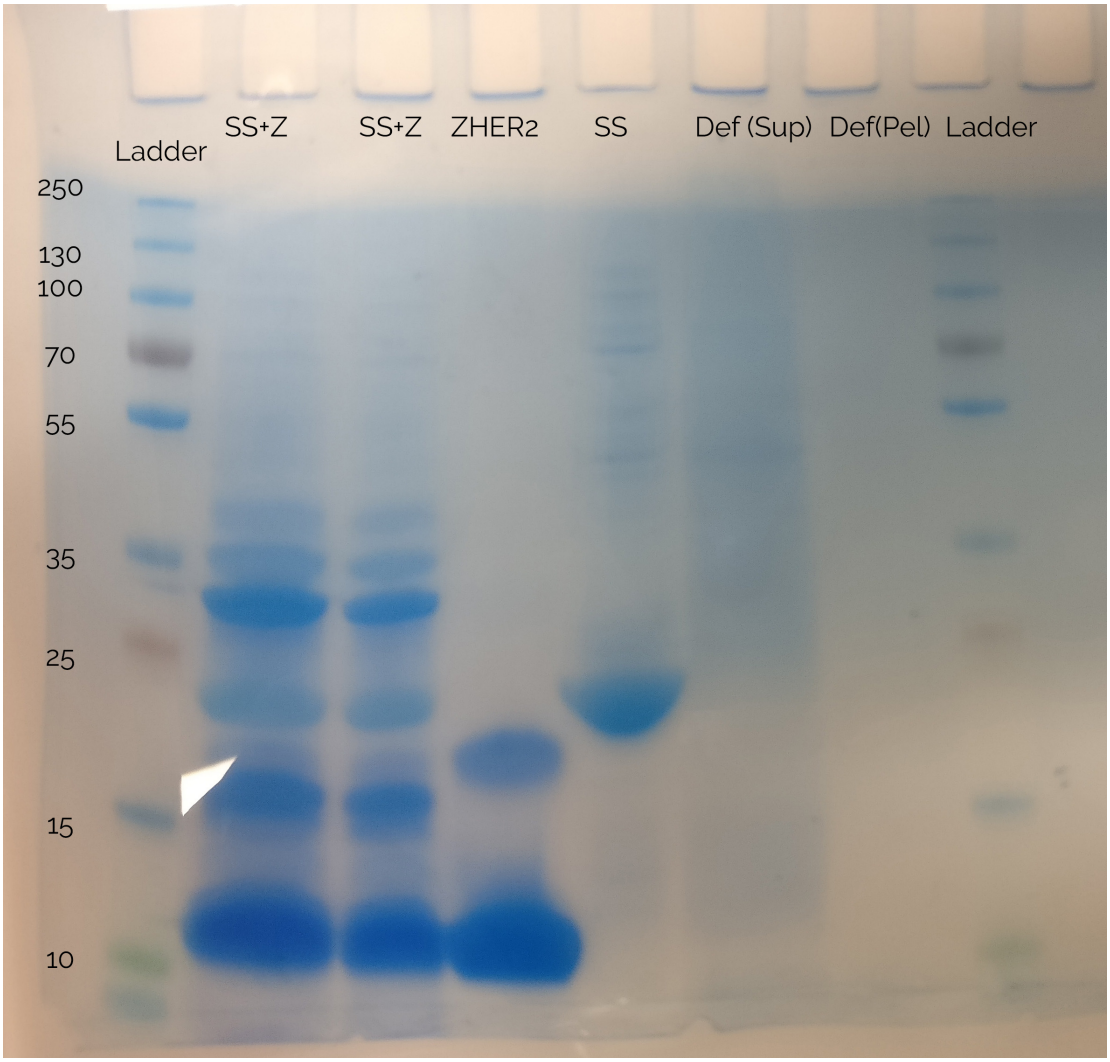

Figure 11: Result of the SDS-PAGE run at 200 V, 3.00 A.

Since the reference in the gel (see Figure 10) is not visible, the spider silk-defensin fusion sample was cross-referenced with another SDS-PAGE made earlier with a non-fused spider silk protein sample (see well labeled "SS" in Figure 11). There, the band corresponding to spider silk protein is clearly at a size of approximately 23 kDa, the expected size of the spider silk protein. From this, the conclusion can be made that the conjugation of recombinant produced defensin, with attached His_6-tag and LPETGG cognition motif, to spider silk protein using Sortase A, was successful.

Discussion and Troubleshooting

Due to time limitations, no further analysis of the spider silk-defensin complex was conducted. A Kirby-Bauer test and a biofilm assay would have been performed to investigate the bactericidal, and biofilm dispersal and inhibition activity, respectively.